CHAPTER SIXTEEN

A LOW-COST TECHNOLOGY FOR ENTOMOPATHOGENIC NEMATODE LARGE-SCALE PRODUCTION

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ABSTRACT

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* may provide a valuable alternative to chemical insecticides. The characteristics that make them excellent biopesticides include their wide host spectrum, the ability to search for and kill hosts rapidly, and their high virulence and reproductive rates. Furthermore, they are considered environmentally safe. The major constraint to overcome before the onset of commercialisation is their mass production. Entomopathogenic nematodes are currently mass-produced *in vivo* or *in vitro*, either in solid culture or in liquid cultivation. An overview of these mass production methods and an analysis of three different bioreactor designs are presented. The progress achieved in liquid culture due to an improvement on sexual contact between adults (better mixture of the solid phase), which results in higher yields (RF), as compared with those reported before, is demonstrated. This improvement in the area of bioreaction engineering allowed these biopesticides to become more competitive compared to chemical insecticides. However, further technological advances and biological studies towards a better understanding of physiology and genetics of the complex nematode-bacterium are still required.

Keywords: biological control, biopesticide, entomopathogenic nematodes, mass production, liquid cultivation, airlift bioreactor

INTRODUCTION

Nematodes are cylindrical roundworms that are unsegmented and lack appendages. They can be free-living, predaceous, or parasitic. Many of the parasitic species cause

disease to plants, animals and humans. However, other species are beneficial in attacking insect pests. Among them, entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* emerged as excellent candidates for biological control of insect pests.

Entomopathogenic nematodes form dauer (enduring) juveniles (DJs) which are morphologically and physiologically adapted for long-term survival in the soil (Figure 16.1). These DJs carry, in the anterior part of the intestine, an associated bacterium; *Xenorhabdus* spp. and *Photorhabdus* spp. in *Steinernema* and *Heterorhabditis* respectively.

When infective DJs locate a susceptible host insect, they enter through the natural body openings (mouth, spiracles and anus) or directly through the insect cuticle and invade its hemocoel, atfer which they release the bacteria. The bacteria multiply, killing the insect by septicaemia within 24–48 h of infection. The nematodes feed on the host tissue and cells of the symbiotic bacteria, develop into adults, mate, and reproduce inside the host, often for multiple generations. When host nutrients are depleted, DJs are produced and, upon leaving the dead insect, seek and infect new hosts.

Thus, entomopathogenic nematodes are a nematode-bacterium complex. In this complex the relationship between the nematode and the bacterium is symbiotic because nematode growth and complete development depend upon conditions established by the bacterial action and, as bacteria lack invasive capacity, they depend upon the nematode to enter the insect hemocoel, thereby causing infection.

Figure 16.1 *Steinernema carpocasae* dauer juvenile.

Nematodes as Bioinsecticides

Since the 1930s steinernematids have been used as agents against agricultural pests. Particular characteristics of these entomopathogenic nematodes are their wide host spectrum, which include the majority of insect orders and families, their ability to search for and kill hosts rapidly, and their high virulence and reproductive rates. Moreover, the steinernematids are considered environmentally safe and non-toxic to mammals, being, thus, exempt from registration in the U.S. as well as in the majority of the E.U. countries.

As DJs spend their entire life cycle in the soil, they are ideal parasites of insects living in the soil and in cryptic habitats. Thus, the market for these nematodes are predominantly soil-dwelling insect pests that attack crops such as corn, rice, vegetables and ornamentals, where nematodes can be used alone or combined with other agents. Examples of commercial nematode-based products that have been successfully used are: *Othyorinchus sulcatus* (black vine weevil), *Scapteriscus vicinus* (tawny mole crickets); *Fumibotys fumalis* (the mint root borer); *Chrysoteuchia topiaria* (the cranberry girdler); *Pachnaeus litus* (Florida citrus); *Diabrotica virgifera virgifera* (western corn rootworm); *Popillia japonica* (Japanese beetle); *Bradysia* spp (fungus gnat); and several *Noctuidae* in different crops.

Entomopathogenic complex insecticidal proteins have been sought since the 1990s. The infective DJ is able to invade insect tissues probably due to a combined mechanical and enzymatic activity and it has been proved that *S. carpocapsae* infective secretes, in artificial growth media, proteases shortly after its development starts. These proteases cause the histolysis of the insect mid-gut, suggesting that they participate in the invasive process (Simões *et al.*, 2000). Once inside the host hemocoel, *S. carpocapsae* infectives are able to escape insect defences, and to produce several other proteins, that cause the insect's immuno-depression (Götz *et al.*, 1981) and are toxic (Burman, 1982; Boemare *et al.*, 1982). Some of these proteins were obtained *in vitro* and it was shown that they cause mortality on insects (Simões & Rosa, 1996).

Xenorhabdus nematophilus are highly pathogenic to insects. They multiply quickly in insect hemocoel causing bacteremia and releasing several enzymes and other proteins that cause toxaemia (Forst *et al.*, 1997). Recently, it has been suggested that two proteases released by the bacteria are involved in the pathogenic process.

Why Mass Produce Entomopathogenic Nematodes?

The main impetus for the commercialisation of insect biocontrol agents is the perception that regulatory pressures imposed by governmental agencies and public opinion will increase. Both demand insecticides with low toxicity and short-term persistence, low mobility in the soil to prevent ground-water contamination, limited effects on non-target organisms and a far greater reduction on chemical input into the environment. These prerequisites have reduced the chance of new active insecticides to be developed and successfully registered.

Entomopathogenic nematodes, because of all the characteristics stated above, can alternatively be used to control soil insects and even have substantial advantages over chemical insecticides.

NEMATODE MASS PRODUCTION IN BIOREACTORS

Commercial exploitation of entomopathogenic nematodes, as of any biological control agent, depends on the selection of an adequate lineage and on the ability of industry to develop and commercialise *Steinernema* as a biopesticide. Until now, one of the most important limiting factors for the development of nematode-based products has been the inability of industry to develop an adequate productive process in order to obtain an economy of scale.

Over the past twenty years significant progress in our understanding of the nutritional requirements of entomopathogenic nematodes resulted in the development of mass production methods. Nowadays, and depending on the objective, three production systems are currently used: *in vivo*, solid culture and liquid cultivation.

In Vivo Production

The first fully operational technique of mass production of *S. carpocapsae* was began in the United States (Dutky, 1964) and is based on parasitism of the greater wax moth, *Galleria mellonella*, by the nematode. It is a simple technique but the cost of production is high and it does not allow an economy of scale. In fact, the duplication of the production capacity requires the duplication of the area and the duplication of the capital. In addition, since the process requires a lot of hand labour, if it is not automated, the costs linearly increase with time. Moreover, the process requires the parallel multiplication of an insect host, which, in turn, also does not allow an economy of scale either. As important as the above aspects is the absence of an economy of quality as the production scale is increased. In fact, the opposite occurs: as scale increases, the *in vivo* nematode production is much more sensitive to insect disease outbreaks which are so characteristic of mass rearing.

In Vitro Production

The limitations of *in vivo* production, coupled to the finding that entomopathogenic nematodes could grow in artificial medium with their symbiotic bacteria, the need for larger scale and more economical methods and the recent advances in the understanding of the nutritional requirements of steinernematids, resulted in the development of *in vitro* mass production methods.

Solid culture

Bedding (1981, 1984) developed a system based on the use of small pieces of inorganic support (shredded sponge) soaked with protein-rich media (preferably chicken offal). This substrate was autoclaved, inoculated with the symbiotic bacterium and incubated. After a two-day incubation, juveniles were inoculated and incubated.

This system promotes the aeration of the environment in nematode culture and a large area to volume ratio for the reproduction and development of the nematode/bacterium complex allowing yields of approximately 10×10^9 dauer juveniles per 3-Kg bag of media.

The "Bedding-System" is a flexible method with low capital costs and it does not need specialised workmanship; therefore, it is attractive for many American, European and Chinese companies. It represents a significant improvement in the productive process of entomopathogenic nematodes. However, this system has some important disadvantages: its sensitivity to contamination, the need for large climate-controlled space for incubation, the considerable amounts of water necessary for downstream and the huge amount of solid waste material to be disposed of. In a scale-up model, Friedman (1990) reported that the "Bedding-System" was economically feasible up to a production level of approximately 10×10^{12} DJs per month. For nematode production beyond this level, labour costs increase significantly suggesting that more advanced technology is needed to support larger scale production of entomopathogenic nematodes.

Liquid culture

Nowadays, it is consensual that the technology of cultivation in liquid medium could be the best method for the mass production of these biopesticides, as the maximisation of volumetric productivity allows the minimisation of the capital invested (Bonifassi & Neves, 1990, Friedman, 1990; Ehlers, 1996). On the other hand, liquid medium culture simplifies the "scale-up" and downstream processing thus allowing their cost reduction.

The first reference to the culture of *Steinernema* in liquid medium goes back to the 1940s (Glaser, 1931, 1940). Since then, the growth of nematodes in liquid medium has been firmly established. Several studies showed that the biological and physiological needs of the nematodes could be satisfied in liquid medium; first in small volumes (Stoll, 1961; Jackson, 1973; Buecher & Popiel, 1989) and later in larger volumes (Georgis & Hague, 1991).

Pace *et al.* (1986) obtained concentrations of 90×10^3 DJs/ml in a 10L stirred tank bioreactor. Friedman *et al.* (1989) reported concentrations above 95×10^3 DJs/ml in an airlift bioreactor. Despite these progresses, the production costs are still high, thereby limiting the use of these biopesticides to control insect pests in high-value crops. Therefore, it is crucial to improve the biotechnological process acting at the level of the bioreaction engineering and more specifically in the bioreactor design.

BIOREACTORS FOR ENTOMOPATHOGENIC NEMATODE PRODUCTION

Steinernematid mass production in liquid culture is a highly complex process when compared with the liquid culture of yeast or bacteria. Indeed, we are discussing a mixed multiphased system. In this system the solid phase is not homogeneous. Furthermore, the reproduction of these organisms is only sexual, involving therefore a correct blending between sexually dimorphic males and females, with different lengths and densities (Neves *et al.*, 1996), and these adults coexist with several other developmental stages.

To this solid phase, we must add a liquid phase where the nutrients are dissolved and, because nematodes are aerobes, abundant aeration to the liquid medium, where the solubility of oxygen is very low. Thus, in the case of liquid culture of entomopathogenic nematodes we are compelled to take into account at least two solid phases – one for each sex, if one disregards the influence of the intermediate juvenile

nematode stages – a liquid phase and a gaseous phase. The need for an appropriate mating rate coupled with the different shear sensitivity of all stages present in the bioreactor lead to the conclusion that traditional stirred bioreactors are definitely not appropriate for achieving high nematode productivities. A multiphase bioreactor is therefore needed.

Airlift systems are becoming everyday more important (Siegel & Robinson, 1992) and should be considered as an alternative to the stirred bioreactor. In an airlift system, the fluidisation of solids is not a direct consequence of the bubbling of gas, but rather due to the liquid circulation within the bioreactor. This system creates an environment of relatively low shear forces ideal for the culture of sensible cells, e.g. those of mammals, vegetables and nematodes, and is especially appropriate for three-phase systems (Kargi & Cervoni, 1983; Kloosterman & Lilly, 1985; Kennard & Janekeh, 1991). There should be a potential application of airlift systems in three-phase processes where gas, liquid and solids must be brought into contact, which is the case for nematode cultivation.
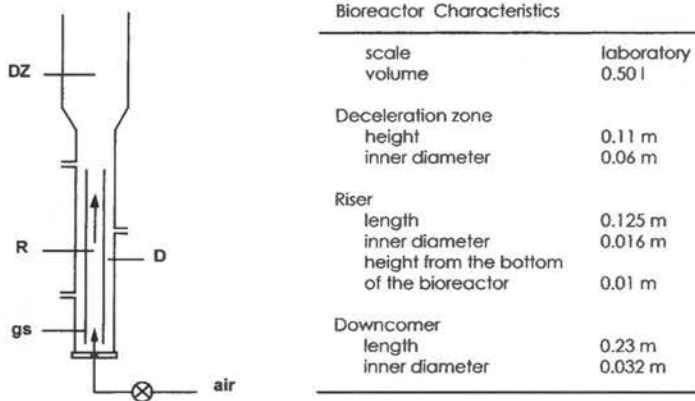
Bioreactor Design

The design of a bioreactor – the heart of a cultivation process where a favourable environment is maintained – must satisfy the biological and technological requirements of the process in cause. Pace *et al.* (1986) and Friedman *et al.* (1989) verified that aeration was the most difficult requirement to meet and that shear sensitivity was the most significant limitation for efficient nematode production. Neves *et al.* (1998) pointed out the low copulation rates, resulting from high aeration and agitation rates, as a factor of great importance in the low yields obtained. Due to the particular characteristics of entomopathogenic nematodes – namely shear sensitivity and sexual dimorphism – mass production in liquid cultivation cannot follow the traditional concepts normally used in the stirred bioreactors. Two major factors have been mandatory in the choice of the bioreactor design: i) an adequate blending of the two sexes; and ii) an appropriate oxygen supply with an acceptable shear stress.

The following examples illustrate the use of several airlift configurations in the liquid cultivation of entomopathogenic nematodes.

Internal-loop airlift

An internal-loop airlift bioreactor, was constructed in Perspex (Figure 16.2). The total height was 0.30 m, a downcomer (D) with 0.23 m of height and an inside diameter of 0.032 m, containing a concentric 0.125 m high and 0.016 m diameter draft tube (R). The ratio of the cross–sectional area of the riser to the down-comer (AR/AD) was 0.28. The top section was of the one of cylindrical conical type. The angle of the conical sector with the main body of the reactor was 45° and the height and diameter of the cylindrical part were, respectively, 0.11 m and 0.06 m.
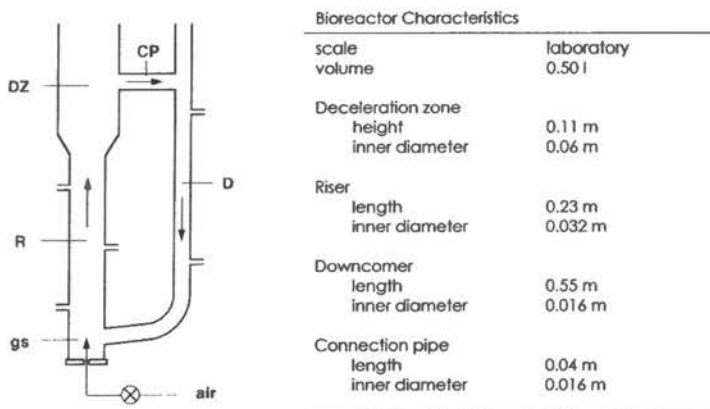
The air was injected beneath the riser annulus by means of a nozzle injector with 0.5 mm diameter.

Bioreactor Characteristics

scale	laboratory
volume	0.50 l
Deceleration zone	
height	0.11 m
inner diameter	0.06 m
Riser	
length	0.125 m
inner diameter	0.016 m
height from the bottom of the bioreactor	0.01 m
Downcomer	
length	0.23 m
inner diameter	0.032 m

Figure 16.2 Schematic diagram of the internal-loop bioreactor. R (riser); D (down-comer); DZ (deceleration zone); gs (gas sparger).

Conventional external-loop airlift

The schematic diagram of the conventional external–loop airlift is shown in Figure 16.3. It consisted of a Plexiglas bioreactor and was composed of a riser (R) with an inside diameter of 0.032 m and a downcomer with an inside diameter of 0.016 m. The ratio of the cross–sectional area of the downcomer (D) to the riser (AD/AR) was 0.28. The top of the riser – deceleration zone (DZ) – was of cylindrical conical type, to facilitate the solid phase (nematodes) deceleration. The height and diameter of this enlarged part were 0.11 m and 0.06 m, respectively. All other dimensions are shown in Figure 16.3. The riser, downcomer and connecting pipes were tubular and, in order to avoid a settling zone, the down-

Bioreactor Characteristics

scale	laboratory
volume	0.50 l
Deceleration zone	
height	0.11 m
inner diameter	0.06 m
Riser	
length	0.23 m
inner diameter	0.032 m
Downcomer	
length	0.55 m
inner diameter	0.016 m
Connection pipe	
length	0.04 m
inner diameter	0.016 m

Figure 16.3 Schematic diagram of the conventional external-loop bioreactor. R (riser); D (down-comer); DZ (deceleration zone); gs (gas sparger); CP (connection pipe).

most connection pipe between the riser and down-comer was inclined. The airlift vessel contained a non-aerated working volume of 0.5 L. Air was sparged into the bioreactor through a nozzle injector with a 0.5 mm diameter placed beneath the riser.
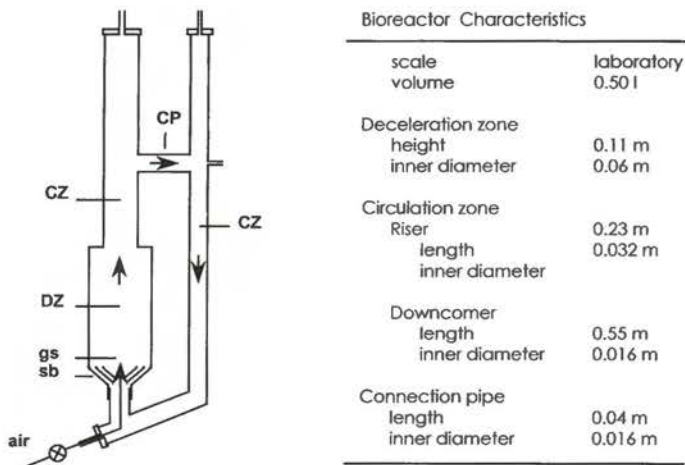
Non conventional external-loop airlift

The most important design characteristics are shown in Figure 16.4. The bioreactor included a circulation zone (CZ) and a deceleration zone (DZ). The circulation zone was composed by two connected pipes: one of 0.23 m and 0.032 m and the other of 0.55 m and 0.0016 m, height and diameter respectively. The height of the deceleration zone was 0.11 m and the diameter 0.06 m. This zone had 2 concentric bulkheads to decrease the liquid circulation velocity. To avoid solids settling, the connection pipe between the circulation zone and the deceleration zone was inclined.

Experimentation

The experimentation included i) the hydrodynamic characterisation of each bioreactor (measurement of mixing parameters and of oxygen mass transfer) and ii) the determination of the bioreactors efficiency concerning adult distribution, mass transfer and yields.

With the internal-loop airlift bioreactor, the circulation and hence the distribution of both adult forms was uniform across the different sections of the bioreactor. One of the basic premises was the improvement in contact between the two sexes: the experimental results obtained with this configuration proved its inadequacy to promote an increase in the mating rate.

Figure 16.4 Schematic diagram of the experimental non-conventional external-loop bioreactor. CZ (circulation zone); DZ (deceleration zone); CP (connection pipe); gs (gas sparger); sb (setting baffles).

The experiments with the external-loop airlift bioreactor clearly showed that male and female distribution was affected differently (Figure 16.5) (Neves, 1994). The distribution of males (Figure 16.5A) was independent of the airflow rate, whereas the female distribution pattern showed a strong dependence on the aeration (Figure 16.5B). This is not surprising and should be expected considering the differences between males and females in the physical properties concerned, namely size and density. Indeed, *S. carpocapsae* females are much bigger than males and have a different density (Neves *et al.*, 1996b). These differences led to different sedimentation rates and, under different airflow rates, to different distribution patterns. At 0.05 vvm, the liquid circulation velocity (0.30 cms^{-1}) was slightly lower than the sedimentation rate of the nematode females in Tyrodes' solution (0.37 cms^{-1}). Under these conditions, it was possible to retain 60.6 % of the females in the deceleration zone. When the airflow rate was 0.09 vvm, females tended to distribute evenly between the different sections of the bioreactor and this tendency became more prominent at 0.15 vvm. At this airflow rate, the liquid circulation velocity increased to 0.62 cms^{-1} and the percentage of females retained in the deceleration zone decreased to 38.9%. In general, it may be concluded that high airflow rates bring about a uniform nematode distribution.
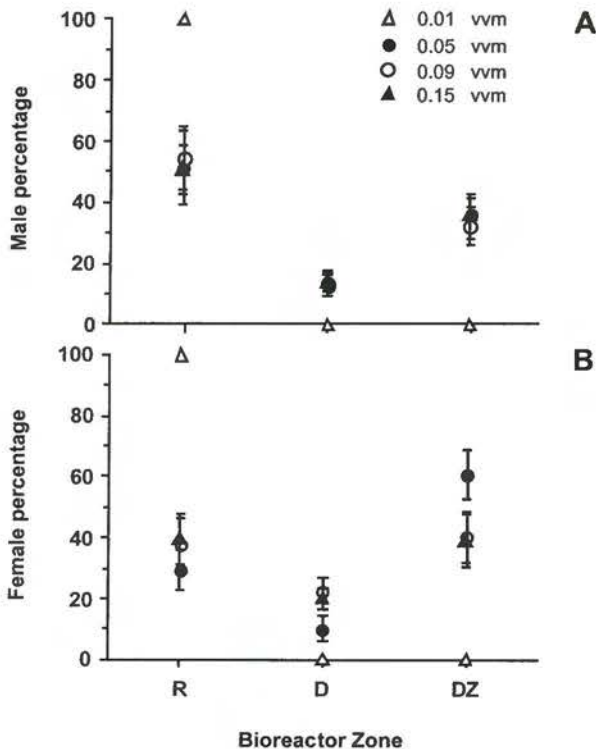
Figure 16.5 Distribution of males (A) and females (B) in the conventional external-loop bioreactor at different airflow rates.

The mass transfer coefficient ($k_L a$) was measured for different airflow rates. Increasing the airflow rate increased the $k_L a$, with 9.3 h^{-1} at 0.05 vvm as the highest value. This value is insufficient to fulfil the oxygen requirements of nematode cultures (Neves *et al.*, 1996a) and lower than the one reported by other authors (Chisti, 1989) for identical systems. The small dimensions of the bioreactor and the design of the air distribution system could account for the low $k_L a$ observed.

Concerning yields obtained after 15 days operation, at 0.05 vvm the DJs production in the bioreactor attained 30×10^6 (60×10^3/ml). This value underwent a 39% reduction when an airflow rate of 0.15 vvm was used. In these conditions the maximum number of DJs obtained was just 19×10^6 (38×10^3/ml).

It is important to notice that the above set of results shows the heterogeneity of adult distribution pattern when different airflow rates are used. These experimental observations appear to agree with similar findings by Siegel *et al.* (1989) and Assa and Bar (1991). The solid phase heterogeneity can be very important when this solid phase is composed of particles with different physical properties such as nematodes in which males and females have different sizes and densities (Neves *et al.*, 1996b). In order to maximise egg productivity, which after hatching will give rise to juveniles, as many females as possible must be fertilised. Therefore, it is important to concentrate females in one region of the bioreactor, so that mating between the retained females and the circulating males will be favoured.

On the other hand, as shown in Figure 16.5, with the exception of 0.01 vvm the region where the maximum number of females is observed – always above 40% of the total – corresponds, in all aeration rates, to the deceleration zone. Furthermore, the aeration which maximises the number of males is in DZ is 0.05 vvm. On the contrary, for 0.15 vvm, a smaller number of males will be present in DZ. Thus, it is quite reasonable to admit that the encounters between males and females are favoured by an aeration rate of 0.05 vvm. Since it was shown that the yield was at its maximum at 0.05 vvm, it is also probable that mating occurs mainly in DZ.

Analysis of the data obtained with the conventional external-loop airlift bioreactor showed that it was possible to improve the mixing and increase $k_L a$ and consequently increase yields (exploiting the particularities of the solid phase, namely the density differences between adults) by coupling a decanter to the bioreactor. Therefore, a non-conventional external-loop airlift bioreactor was designed. In this bioreactor, the enlarged part of the riser – deceleration zone – acted as a "gynaeceum" where the mating rate was enhanced.

The experimental results obtained with the novel bioreactor confirmed expectations. For airflow rates of 0.3 vvm, male concentrations in the deceleration zone were close to 60% (Figure 16.6A). For higher airflow rates, the male distribution was uniform. Similarly, at airflow rates between 0.3 and 0.8 vvm it was possible to retain 70% of females in the deceleration zone (Figure 16.6B). For higher airflow rates, the female DZ concentration was reduced and females tended to uniformly distribute across all sections of the bioreactor.

It is important to notice that the airflow rates used in this reactor were tenfold greater than on the other bioreactors used. As a consequence, the volumetric oxygen mass transfer coefficient increased significantly, varying between 40.9 h^{-1} and 138 h^{-1} for 0.3 vvm
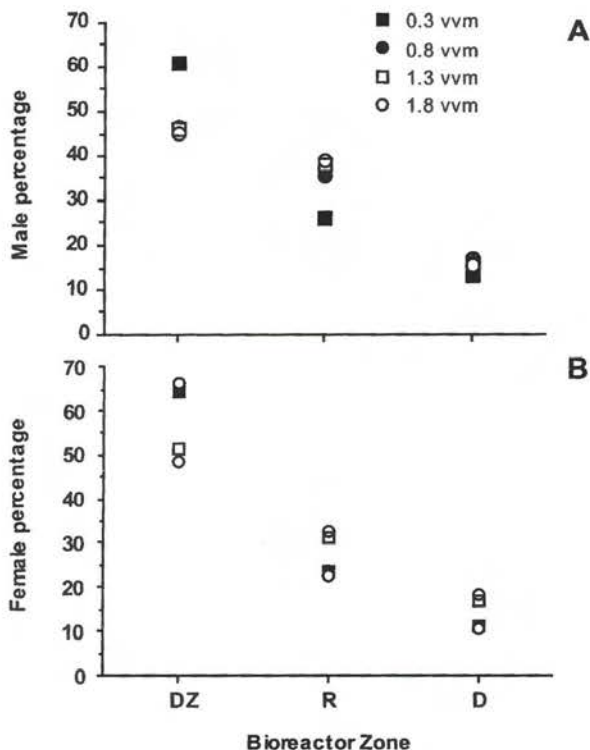
Figure 16.6   Distribution of males (A) and females (B) in the non-conventional external-loop bioreactor at different airflow rates.

and 2.8 vvm respectively. For 0.8 vvm, the airflow rate which promotes the biggest concentration of females (70%), the $k_L a$ was 50.9 h^{-1}. Under these conditions, after 15 days of operation, it was possible to obtain a total number of DJs of 59×10^6 $(97,5 \times 10^3/ml)$ (Neves *et al.*, 1998).

CONCLUSIONS

Recent developments in bioreactor design have attempted to either address some of the limitations of existing bioreactors or open new avenues in bioprocessing. The further development of innovative bioreactor designs remains a high priority, since a single bioreactor configuration will never provide universal solution. In many instances, progress in a reactor design will require preliminary advances in the biological fundamentals. Conversely, many questions addressed to biologists by bioprocess engineers may provide new fields for fundamental research. The answers found in the meantime may then give rise to a more rational, creative and focused approach to bioreactor design.

The data obtained in this work show that the airlift system proposed is rather more efficient than those reported before (e.g., Pace *et al.*, 1986 and Friedman *et al.*, 1989) (Table 16.1). The advantage of the proposed bioreactor design results from the improvement of sexual contact between adults by creating a zone of low liquid velocity where a high concentration of females is maintained, thus increasing the mating opportunities with males that circulate in the bioreactor. In fact, although uniform distribution of the solid and liquid phases throughout the airlift vessel is generally sought, it is sometimes desirable to create non-homogenous distribution patterns of the solid phase in particular cases. By associating the differences in the physical properties of the components of the solid phase – namely, male and female nematodes – with an adequate design for the bioreactor, it was possible to develop a more efficient system for nematode production in submerged cultures.

The improvement obtained with this design becomes more clear if the yield achieved, i.e., the reproductive factor (RF = final concentration/inoculum concentration), is compared with those obtained with other *S. carpocapsae* production systems. As may be seen in Table 16.1, with the novel bioreactor design, a 2-fold improvement in yield was achieved. The data also show that the aeration rate could not be directly responsible for production differences, since a lower aeration rate induced a higher production. The significantly higher yield must be associated with an increase in the mating rate due to a lower liquid circulation velocity.

In spite of these data, there are still some questions to be answered, which may bring about a great impact. Coupled with the advances in bioreaction engineering, particularly in bioreactor design and process control, optimisation of the cultivation environment (physical and chemical) is of utmost importance for maximising development, copulation, fecundity and formation of dauer juveniles.

Organism development is also a key area in liquid culture. Thus, a better understanding of both nematode and bacterial biology, especially genetics and physiology, is required.

The process of exit from enduring infective stage (recovery) is crucial. The importance of recovery lies on its influence on population dynamics, the duration of the cultivaton process and, certainly, on the final yield. The percentage of in vitro culture recovery is very low, but so far little is known about the factor(s) that control this process, urging the need to better understand its regulatory mechanisms.

Table 16.1 Yield comparison among different bioreactor designs for production of *Steinernema carpocapsae* in liquid medium

Bioreactor	Duration (Days)	Inoculum (DJs/ml)	Final (DJs/ml)	Rf
stirred tank bioreactor (a)	20	2000	90000	45
internal-loop airlift bioreactor (b)	15	1000	95000	95
internal-loop airlift bioreactor (c)	15	500	60000	120
external-loop airlift bioreactor (c)	15	500	97500	195

Rf (reproduction factor) = Final concentration/Inoculum concentration.
(a) Pace *et al.* (1986); (b) Friedman *et al.* (1989); (c) our work

On the other hand, the importance of *Xenorhabdus* spp. in nematode production is well-documented (Akhurst & Boemare, 1990). The bacterium has two phases, the primary and secondary, the primary being the only phase that supports greater nematode production. Both, genetic and physiological approaches must be used in order to know i) the dynamics of the two phases in the bioreactor; ii) the stability of the primary form of the bacteria; and iii) the interaction between bacteria and nematodes.

In conclusion, the progress achieved in liquid cultivation of steinernematids is nothing but another step forward to make entomopathogenic nematodes competitive with chemical insecticides in medium and high-value crops on the basis of cost/benefit ratio and ease of application.

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